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PROTEIN KINASES

This invention is related to the one or more inventions described in British Patent Applications Nos. 9224057.1, filed 17th November 1992, and 9304677.9 and 9304680.3, both filed 8th March 1993. In particular, this invention relates to nucleotides, proteins obtained by expression therefrom and antibodies raised to peptides derived from the sequence, e.g. by the means described in the Application No. 9304680.3.

A particularly important aspect of the invention is based on the discovery that a member of the serine/threonine kinase family is a TGF-ß-type I receptor. It has not previously been possible to isolate this receptor, to allow determination of its sequence or of the corresponding gene, as the expression levels of the receptor are very low.

The isolation of the nucleic acid molecule which codes for the TFG-ß-type I receptor allows it to be expressed at high enough levels in cell lines, e.g. for use in antagonist/agonist screening. It also allows the production of the protein, and portions of the protein, e.g. the extracellular domain, for therapeutic, diagnostic and other commercial purposes.

Summary

A cDNA clone encoding a 53 kd serine/threonine kinase receptor with an overall structure similar to that of the type II receptor for TGF-β was obtained. ^{125}I-TGF-β1 bound to porcine aortic endothelial cells transfected with the cDNA, and formed a cross-linked complex of 70 kd, that is characteristic of a TGF-β type I receptor. Immunoprecipitation of the cross-linked complexes by antibodies raised against the cloned receptor revealed the 70 kd complex as well as a component of 94 kd, which is the expected size of a TGF-β type II receptor complex. The 70 kd complex has properties of the TGF-β type I receptor, i.e. a small molecular weight shift after enzymatic deglycosylation and susceptibility to DTT treatment. These results indicate that the cDNA clone encodes a TGF-β type I receptor, and suggest that signal transduction by TGF-β involves the heteromeric complex of two different serine/threonine kinase receptors.

Introduction

Transforming growth factor-β (TGF-β) is a family of multifunctional proteins that act on many different types of cells (for reviews see Roberts and Sporn, 1990; Moses et al., 1990). TGF-β was first identified as a 25 kd homodimer, that stimulated the growth of normal rat fibroblasts in soft agar culture (Assoian et al., 1983). However, it was later shown to be a potent growth inhibitor for most cell types (Moses et al., 1985). TGF-β also has many other biological effects, including regulation of cell differentiation, stimulation of extracellular matrix synthesis, and modulation of the immune response. Three different isoforms of TGF-β, denoted TGF-β1, -β2, and -β3, with similar but not identical biological activities (Ohta et al., 1987; Cheifetz et al., 1990), have been identified in various mammalian tissues and cells. TGF-βs belong to a larger family of structurally related proteins, which includes activins and inhibins (Vale et al., 1990), bone morphogenetic proteins (BMPs) (Wozney et al., 1988), and Müllerian inhibiting substance (Cate et al., 1986). The proteins of the TGF-β superfamily have a wide variety of biological activities and play important roles in the morphogenesis, e.g. during different stages of development (Akhurst et al., 1991; Lyons et al., 1991).

TGF-βs exert their effects through binding to specific cell surface receptors. By affinity labeling and cross-linking by radioiodinated TGF-βs, a number of TGF-β receptors (or binding proteins) have been identified, including type I (53 kd), type II (75 kd) and type III receptors (or betaglycan, 300 kd), which are found in most cells (reviewed in Massagué, 1992; Lin and Lodish, 1993; Miyazono et al., 1993). The type I and the type II receptors are the most important for signal transduction (Segarini et al., 1989; Boyd and Massagué, 1989; Laiho et al., 1990). The recent cloning and characterization of the TGF-β type II receptor (Lin et al., 1992) and the type III receptor (López-

Casillas et al., 1991; Wang et al., 1991; Morén et al., 1992), have improved the understanding of the signal transduction of TGF-β.

The TGF-β type III receptor is a membrane proteoglycan lacking cytoplasmic protein kinase domain. The transmembrane and intracellular parts of the type III receptor are very similar to the corresponding region of endoglin, a 180 kd dimeric protein, which is expressed in endothelial cells and in the mesangium of the kidney (Gougos and Letarte, 1988; 1990). Endoglin binds TGF-β1 and -β3, but not TGF-β2, with high affinities, whereas the type III receptor binds all three mammalian isoforms of TGF-β (Cheifetz et al., 1992). The type III receptor and endoglin may be indirectly involved in the signal transduction, e.g. by presenting the ligands to the type II and type I receptors (Wang et al., 1991; Cheifetz et al., 1992).

The TGF-β type II receptor cDNA was obtained by an expression cloning strategy (Lin et al., 1992). Similar to the activin type II and type IIB receptors (Mathews and Vale, 1991; Attisano et al., 1992; Mathews et al., 1992), the TGF-β type II receptor has a serine/threonine kinase domain in its cytoplasmic portion. Autophosphorylation on serine and threonine residues of the TGF-β type II receptor kinase has been demonstrated using a bacterial fusion protein (Lin et al., 1992). A transmembrane serine/threonine kinase had previously been found in the Daf-1 protein (Georgi et al., 1990), which is involved in the dauer larva development. These results suggest that receptor serine/threonine kinases form a new receptor family, which may include the receptors for the proteins in the TGF-β superfamily.

The TGF-β type I receptor has been shown to be a 53 kd glycoprotein using affinity cross-linking techniques (Cheifetz et al., 1988a; Cheifetz and Massagué, 1991). Studies using chemically-induced TGF-β receptor mutant cells indicated that both type I and type II receptors are indispensable for TGF-β signaling (Boyd and Massagué, 1989; Laiho et al., 1990); the type II receptor is needed for the binding of TGF-β to the type I receptor, and the type I

receptor is required for the signal transduction induced by the type II receptor (Wrana et al., 1992). However, the structure of the type I receptor has not been fully determined.

Here, we report the isolation and characterization of a human cDNA clone encoding a novel 53 kd putative serine/threonine kinase receptor. The biochemical and biological properties of the cloned receptor indicate that it represents a type I receptor for TGF-β.

Results

Cloning and Analysis of a TGF-β Type I Receptor cDNA

In order to identify novel serine/threonine kinase receptors, a PCR-based approach was used with the prediction that these receptors share sequence similarities to each other (ten Dijke et al., 1993). PCR was performed using cDNA from human erythroleukemia (HEL) cells and degenerated primers that were based on conserved regions in the serine/threonine kinase receptors. The primers used were derived from subdomain II (nomenclature according to Hanks et al., 1988), which includes the lysine residue involved in ATP binding, and from subdomain VIII, which is suggested to determine the hydroxyamino acid specificity for serine and threonine residues rather than tyrosine residues (Hanks et al. 1988). Several different PCR products were obtained, including the TGF-β type II receptor and the activin type II receptor. One PCR recombinant, 11.1, encoded a novel amino acid sequence (not shown), and was therefore used to screen a random primed HEL cell λgt10 cDNA library. Screening of about 1 x 10^5 independent clones yielded one positive clone, denoted EMBLA, which has an insert size of 5.3 kb with two internal EcoRI sites (Figure 1A). Nucleotide sequencing revealed an open reading frame of 1509 bp, coding for 503 amino acids. The open reading frame is flanked by a

5' untranslated sequence of 76 bp, and a 3' untranslated sequence of 3.7 kb which was not completely sequenced. The nucleotide and deduced amino acid sequences of the EMBLA clone are shown in Figure 1B. The amino acid sequence has a 90% sequence identity to the deduced amino acid sequence of the 11.1 PCR recombinant that was used as a probe for screening of the library. Based on evidence which will be presented below, we conclude that EMBLA encodes a type I receptor for TGF-β. Therefore, the molecule has been called TGF-β type I receptor throughout this communication.

Structure of the TGF-β Type I Receptor Protein

In the 5' part of the open reading frame, only one ATG codon was found, which fulfills the rules for initiation of translation (Kozak et al., 1987). An in-frame stop codon was found at nucleotides (-54)-(-52) in the 5' untranslated region. The predicted starting ATG codon is followed by a stretch of hydrophobic amino acid residues which has the characteristics of a cleavable signal sequence. Therefore, the first ATG codon is likely to be used as a translation initiation site. A preferred cleavage site for the signal peptidase, according to von Heijne (1986), is located between amino acid residues 24 and 25. The calculated molecular weight of the primary translated product of the TGF-β type I receptor without signal sequence is 53,646.

Another hydrophobic region, which represents a putative transmembrane domain (Kyte and Doolittle, 1982), is found at amino acid residues 126-147, followed by basic residues at His-149 and Arg-151, as is common for the cytoplasmic side of transmembrane regions (Weinstein et al., 1982). The extracellular domain has a cysteine-rich region with one potential N-glycosylation site. It has little sequence similarity with the TGF-β type II receptor (Figure 1C), activin type II and IIB receptors, and Daf-1 (less than 17 % amino acid sequence identity). However, a fair alignment of nine of the ten cysteine residues could be performed. The cytoplasmic part has a putative

protein kinase domain, which contains the consensus motifs specific for serine/threonine kinases, and is 41% identical to that of the TGF-β type II receptor (Figure 1B and C, see Discussion).

Similar to the type II receptors for activin and TGF-β, the protein kinase domain of the TGF-β type I receptor is interrupted by short streches of amino acid residues between subdomains VIA and VIB and between subdomains X and XI (Figure 1B and C). Based on the sequence similarity with the type II receptors for TGF-β and activin, the C-terminus of the kinase domain of the TGF-β type I receptor was set at Gln-498, and thus the C-terminal tail of the type I receptor has only 5 amino acid residues, which is considerably shorter than those of the TGF-β and activin type II receptors (more than 23 amino acid residues).

The TGF-β Type I Receptor cDNA Encodes a 53 kd Glycoprotein

The TGF-β type I receptor cDNA was subcloned into the SV40-based expression vector pSV7d and transfected into COS-1 cells. Two days after transfection, the cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine and subjected to immunoprecipitation using an antiserum (VPN), which was raised against a synthetic peptide corresponding to amino acids 158-179 in the juxtamembrane part of the cytoplasmic domain. This region is divergent in sequence between the various serine/threonine kinase receptors (not shown). A component of 53 kd was seen when the samples were analyzed by SDS-polyacrylamide gel electrophoresis (Figure 2). This component was not seen when preimmune serum was used, or when 10 μg of blocking peptide was added together with the antiserum. Moreover, it was not found in samples derived from untransfected COS-1 cells using either preimmune serum or the antiserum. When the immunoprecipitated sample was treated with endoglycosidase F, that hydrolyzes the complex- and high mannose-types of N-linked carbohydrates, the 53 kd band shifted to 51 kd.

The extracellular domain of the TGF-β type I receptor contains one potential acceptor site for N-glycosylation and the size of the deglycosylated protein is close to the predicted size of the core protein.

Binding of ^{125}I-TGF-β1 to the Type I Receptor in Transfected PAE Cells

In order to investigate whether the cloned cDNA encodes a receptor for TGF-β, porcine aortic endothelial (PAE) cells were transfected with a cytomegalovirus (CMV)-based expression vector pcDNA I/NEO containing the cloned cDNA, and analyzed for the binding of ^{125}I-TGF-β1, followed by affinity cross-linking and SDS-gel electrophoresis. ^{125}I-TGF-β1 formed a 70 kd cross-linked compex in the transfected PAE cells (PAE/TβR-I cells). The size of this complex was very similar to that of the TGF-β type I receptor complex observed at lower amounts in the untransfected cells (Figure 3). A concomitant increase of a 94 kd complex, i.e. similar in size to that of a TGF-β type II receptor complex, could also be observed in the PAE/TβR-I cells. A 150 kd complex, which could represent a cross-linked complex between the type I and type II receptors (see Discussion), was also observed in the PAE/TβR-I cells.

In order to determine whether the cross-linked 70 kd complex contained the protein encoded by the transfected cDNA, the affinity cross-linking was followed by immunoprecipitation using the VPN antiserum. A 70 kd cross-linked complex could be precipitated by the VPN antiserum in PAE/TβR-I cells, and a weaker band of the same size was also seen in the untransfected cells (Figure 3), indicating that the untransfected PAE cells contained a low amount of TGF-β type I receptor. The 70 kd complex was not observed when preimmune serum was used (Figure 3), or when immune serum was blocked by 10 μg of peptide (not shown). Moreover, a coprecipitated 94 kd component,

most likely representing a TGF-β type II receptor complex, could also be observed in the PAE/TβR-I cells.

The carbohydrate content of the TGF-β type I receptor was characterized by deglycosylation using endoglycosidase F on cross-linked and immunoprecipitated ^{125}I-TGF-β1 receptor complexes from the PAE/TβR-I cells. The type I receptor cross-linked complex shifted from 70 kd to 66 kd, whereas that of the putative type II receptor shifted from 94 kd to 82 kd (Figure 4A). The observed larger shift of the putative type II receptor band compared with that of the type I receptor band is consistent with the deglycosylation data of the type I and type II receptors on rat liver cells reported previously (Cheifetz et al., 1988a), and fits well with the facts that the porcine TGF-β type II receptor has two N-glycosylation sites (Lin et al., 1992), whereas the type I receptor has only one (Figure 1B).

Binding of TGF-β1 to the type I receptor is known to be abolished by transient treatment of the cells with dithiothreitol (DTT) (Cheifetz and Massagué, 1991; Wrana et al., 1992). When analyzed by affinity cross-linking, binding of ^{125}I-TGF-β1 to the TGF-β type I receptor, but not to the type II receptor, was completely abolished by DTT treatment of PAE/TβR-I cells (Figure 4B). Affinity cross-linking followed by immunoprecipitation by the VPN antiserum showed that neither the type I nor type II receptor complexes were precipitated after DTT treatment, indicating that the VPN antiserum reacts only with the TGF-β type I receptor. In conclusion, our data show that the VPN antiserum recognizes a TGF-β type I receptor, and that the type I and type II receptors form a heteromeric complex.

Expression of the TGF-β Type I Receptor mRNA In Different Tissues

The TGF-β type I receptor mRNA size and distribution were determined by Northern blot analysis. Figure 5 shows that a 5.5 kb mRNA is expressed in all

human tissues tested. The transcript of 5.5 kb was most abundant in placenta and least in brain and heart. The size of the transcript suggests that the obtained cDNA clone was close to full length.

Discussion

In the present report, we show the structure of a novel putative serine/threonine kinase receptor. The following observations support the notion that the molecule is a type I receptor for TGF-β. i) The size of the receptor immunoprecipitated from metabolically labeled COS-1 cells (Figure 2) or PAE/TβR-I cells (data not shown) is 53 kd, which is consistent with the predicted size of a type I receptor (Cheifetz et al., 1988a). ii) ^{125}I-TGF-β1 forms a cross-linked complex of 70 kd, which can be precipitated by an antiserum against the receptor from PAE/TβR-I cells (Figure 3). This is the expected size for a TGF-β type I receptor complex (Cheifetz et al., 1988a). iii) A complex of 92 kd, i.e. the expected size of a TGF-β type II receptor complex is coimmunoprecipitated with the type I receptor complex (Figure 3), which is consistent with the notion that type I and type II receptors form a heteromeric complex after ligand binding (Wrana et al., 1992). iv) Treatment with endoglycosidase F leads to a small decrease in size of the type I receptor complex, but to a larger decrease of the putative type II receptor complex (Figure 4A), consistent with previous observations on type I and type II receptors (Cheifetz et al., 1988a), and with the facts that the type I receptor has one potential N-glycosylation site, whereas the porcine type II receptor has two (Lin et al., 1992). v) Treatment of PAE/TβR-I cells with DTT leads to loss of binding of ^{125}I-TGF-β1 to type I receptors, whereas the binding to the putative type II receptors is retained (Figure 4B). This is consistent with the previously described properties of the type I and type II TGF-β receptor complexes

(Cheifetz and Massagué, 1991). vi) The distribution of mRNA for the cloned receptor is ubiquitous, as is expected for that of the TGF-β type I receptor.

PAE/TβR-I cells also bind ^{125}I-TGF-β2, but with lower affinity than ^{125}I-TGF-β1; however, ^{125}I-activin was not bound (data not shown). Two other TGF-β receptors with sizes in the same range as the novel serine/threonine kinase receptor have been described. One is the TGF-β type IV receptor (70-74 kd cross-linked complex). However, this receptor is expressed only in the pituitary tumor cells and binds TGF-βs as well as activins and inhibins (Cheifetz et al., 1988b), and it is thus unlikely that it is the same as the molecule described in the present report. The other is a 40 kd component purified from porcine uterus (Ichijo et al., 1991). However, this molecule was recently cloned and was shown to have a different sequence (Ichijo et al., 1993). Based on these observations, we conclude that the cDNA reported in this paper encodes a type I receptor for TGF-β.

The evidence that the coimmunoprecipitated component is the previously cloned TGF-β type II receptor (Lin et al., 1992) is less strict, but its size, susceptibility to endoglycosidase F treatment, and the lack of susceptibility to DTT, support the notion that the coimmunoprecipitated receptor is the type II receptor. A 150 kd complex was also observed in the PAE/TβR-I cells, which was coimmunoprecipitated by the VPN antiserum (Figure 3). It is possible that this component represents a cross-linked complex between the type I and type II receptors. Similar high molecular weight complex was also observed after the transfection of activin type II receptors (unpublished data), which support this notion. Alternatively, the component may represent other types of TGF-β receptors, e.g. endoglin, which is expressed in endothelial cells (Cheifetz et al., 1992).

Apart from the transfected PAE cells, the TGF-β type I receptor could also be immunoprecipitated from wild-type PAE cells (Figure 3) and from human foreskin fibroblasts (data not shown). Interestingly, in the latter cells the

VPN antiserum brought down one of two components in a cross-linked doublet band of the type I receptor, suggesting that there might be more than one molecule that can serve as a TGF-β type I receptor.

Using a PCR-based approach, we recently obtained four additional receptor-like serine/threonine kinases, termed activin receptor-like kinase (ALK)-1 to -4 (ten Dijke et al., 1993). The TGF-β type I receptor described in the present report was originally obtained as a fifth clone (ALK-5). The five ALKs have similar sizes and 60-90% amino acid sequence identities to each other in their kinase domains, whereas they have 37-42% identities to the activin type II and IIB receptors and to the TGF-β type II receptor. Thus, the ALKs form a subfamily among the serine/threonine kinase receptors. Their high sequence similarities and sizes make them interesting candidates for being additional type I receptors for TGF-β1 or for other TGF-β isoforms, activins, inhibins, BMPs or other members of the TGF-β superfamily.

Studies using chemically-mutagenized mink lung epithelial cells, which are resistant to the growth inhibitory activity of TGF-β, have indicated that the type I and type II receptors form a heteromeric complex and are directly involved in the signal transduction of TGF-β (Boyd and Massagué, 1989; Laiho et al., 1990). Two types of TGF-β receptor mutant cells were obtained; the class R mutants showed normal binding to the type II receptor but lacked the binding to the type I receptor, whereas the class DR mutants showed no or anomalous binding to both type I and type II receptors. Mutants that lacked binding to only the type II receptor could not be isolated. Somatic cell hybrids between the class R and DR mutants resulted in the restoration of the type I receptor binding (Laiho et al., 1991), indicating that the type I receptor is present in the class DR mutants, but the type II receptor is needed for the ligand binding to type I receptor. More recently, the formation of a heteromeric complex between the type I and type II receptors was demonstrated by the following observations: i) the type I receptor was coimmunoprecipitated with

the type II receptor, ii) transfection of the type II receptor into class DR mutants rescued the TGF-β binding to the type I receptor and made the cells responsive to TGF-β, and iii) a kinase-defective type II receptor transfected into DR mutants allowed TGF-β to bind the type I receptor, but did not restore the sensitivity to TGF-β (Wrana et al., 1992). The properties of the cloned TGF-β type I receptor described in this study are fully consistent with the previous conclusions regarding the TGF-β type I receptor and the activated TGF-β receptor complex. Thus, formation of the heteromeric complex of two serine/threonine kinase receptors may be an important event for TGF-β signaling in its target cells. Future studies will be aimed at exploring the exact mechanism for activation of the TGF-β receptor complex and to determine the contribution of each one of the two receptors in the activation of downstream components in the signal transduction pathway. Furthermore, it will be important to determine whether the receptor complex is a heterodimer or a heterotetramer of TGF-β type I and type II receptors and whether additional TGF-β receptors, e.g. the type III receptor and/or endoglin, are also part of the complex.

Experimental Procedures

Preparation of mRNA and Construction of a cDNA Library

For construction of a cDNA library, poly(A)+RNA was isolated from a human erythroleukemia cell line, HEL (American Type Culture Collection), by the guanidinium isothiocyanate method (Chirgwin et al., 1979), followed by use of a polyAT tract mRNA isolation kit (Promega). The isolated mRNA was used for the synthesis of random primed (Amersham) cDNA. A λgt10 cDNA library with $1x10^5$ independent cDNA clones was prepared by RiboClone cDNA synthesis system (Promega) and in vitro packaging kit (Amersham).

Generation of a cDNA Probe by PCR

For the generation of cDNA probes by PCR (Lee et al., 1988), degenerated PCR primers were constructed based upon the amino acid sequence similarity between the mouse activin type II receptor (Mathews and Vale, 1991) and Daf-1 (Georgi et al., 1990) in the kinase domains II and VIII. Oligonucleotides were synthesized using Gene assembler plus (Pharmacia-LKB). The sense primer, B3-S, was a 25-mer oligonucleotide (5'-GCG GATCCGT(C/G/T)GC(A/C/T)GT(C/G/T)AA(A/G)AT(A/C/T)TT) derived from the conserved motif (VAVKIF in single letter code) in subdomain II with a 5' BamHI restriction enzyme site. The antisense primer, E8-AS, was a 20-mer oligonucleotide (5'-CGGAATTC(A/G/T)GG(A/G/T)GCCAT(A/G)TA) derived from the conserved sequence (YMAPE) in subdomain VIII with a 5' EcoRI site. PCR was performed in a 100 µl volume using first strand cDNA prepared from HEL mRNA, Taq polymerase (Perkin Elmer Cetus) and the oligonucleotide primers. The following program was used for PCR amplification; first 5 thermal cycles, each composed of 94°C (1 min), 50°C (1 min), 55°C (2 min), 72°C (1 min) followed by 20 thermal cycles of 94°C (1 min), 55°C (0.5 min), 72°C (1 min). A second round of PCR was performed with 3 µl of the first reaction as a template. After 25 thermal cycles, each composed of 94°C (1 min), 55°C (0.5 min), 72°C (1 min), the products with the expected sizes (~460 bp) were purified by agarose gel electrophoresis. The PCR products were ligated into pUC19 (Yanisch-Perron et al., 1985) at BamHI/EcoRI sites, and nucleotide sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia-LKB). One of the PCR recombinants denoted 11.1 showed a novel sequence and was used for isolation of a full-length cDNA.

Isolation and Characterization of a cDNA Clone

The HEL cell cDNA library was screened with the insert of PCR recombinant 11.1 labeled by the Megaprime DNA labeling system (Amersham). Hybridization to nitrocellulose filters (Hybond-C extra, Amersham) was performed in 50% formamide, 5 x SSC (1 x SSC is 15 mM sodium citrate and 150 mM sodium chloride), 50 mM sodium phosphate, pH 6.9, 5 x Denhardt's solution, 0.1% SDS and 0.1 mg/ml salmon sperm DNA at 37°C overnight. The filters were washed in 0.5 x SSC, 0.1% SDS at 55°C three times for 15 min, dried and exposed to Fuji X-ray films. Purification of a positive bacteriophage plaque was performed as described by Sambrook et al. (1989). A clone denoted EMBLA was identified and subcloned into pBluescript SK (Stratagene) and thereafter sequenced on both strands. Compressions of nucleotides were resolved using 7-deaza-GTP (United States Biochemical Corp.).

Antibodies for the TGF-β Type I Receptor

Rabbit antisera against the TGF-β type I receptor denoted VPN were prepared against a synthetic peptide corresponding to amino acid residues 158-179. The peptide was synthesized with an Applied Biosystems 430A Peptide Synthesizer using t-butoxycarbonyl chemistry and purified by reversed phase high performance liquid chromatography. The peptide was coupled to keyhole limpet hemocyanin (Calbiochem-Behring) using glutaraldehyde, as described by Gullick et al. (1985). The coupled peptide was mixed with Freund's adjuvant and used to immunize rabbits.

Transfection of the TGF-β Type I Receptor cDNA into COS Cells

COS-1 cells obtained from American Type Culture Collection were used for transient expression. The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 atmosphere at 37°C. The TGF-β type I receptor cDNA (nucleotides (-76)-

2232), which includes the whole coding region, was cloned into the SV40-based expression vector pSV7d (Truett et al., 1985), and transfected into COS-1 cells by the calcium phosphate precipitation method (Wigler et al., 1979). Briefly, cells were seeded into 6-well cell culture plates at a density of 5 x 10^5 cells/well, and transfected the following day with 10 µg of plasmid. After overnight incubation, cells were washed three times with a buffer containing 25 mM Tris-HCl, pH 7.4, 138 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.6 mM Na_2HPO_4, and then incubated with Dulbecco's modified Eagle's medium containing 10% FBS and antibiotics. Two days after transfection, the cells were used for metabolic labeling and immunoprecipitation.

Establishment of PAE Cell Lines Expressing the TGF-β Type I Receptor

PAE cells were cultured in Ham's F-12 medium supplemented with 10 % FBS and antibiotics (Miyazono et al., 1988). The cDNA for the TGF-β type I receptor was cloned into the human CMV-based expression vector pcDNA I/NEO (Invitrogen). The TGF-β type I receptor expression vector was transfected into PAE cells by electroporation, and after 48 h, selection was initiated by adding Geneticin (G418 sulphate; Gibco/BRL) to the culture medium at a final concentration of 0.5 mg/ml (Westermark et al., 1990). Several clones were obtained, and after analysis by immunoprecipitation using the VPN antiserum, one clone denoted PAE/TβR-I was chosen and further analyzed.

Metabolic Labeling, Immunoprecipitation and SDS-gel Electrophoresis

Metabolic labeling of cells was performed for 6 h in methionine- and cysteine-free MCDB 104 medium with 150 µCi/ml of [^{35}S]methionine and [^{35}S]cysteine (In vivo labeling mix; Amersham). After labeling, the cells were washed with

150 mM NaCl, 25 mM Tris-HCl, pH 7.4, and then solubilized with a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% Trasylol (Bayer) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). After 15 min on ice, the cell lysates were pelleted by centrifugation, and the supernatants were cleared one time with preimmune serum. Samples (1 ml) were then incubated with either 7 µl of preimmune serum or the VPN antiserum for 1.5 h at 4°C. For blocking, 10 µg of peptide was added together with the antiserum. Immune complexes were then given 50 µl of a protein A-Sepharose (Pharmacia-LKB) slurry (50% packed beads in 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.2% Triton X-100) and incubated for 45 min at 4°C. The beads were spun down and washed four times with a washing buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.2% SDS), followed by one wash in distilled water. The immune complexes were eluted by boiling for 5 min in the SDS-sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS) in the presence of 10 mM DTT, and analyzed by SDS-gel electrophoresis using 7-15% polyacrylamide gels (Blobel and Dobberstein, 1975). Gels were fixed, incubated with Amplify (Amersham) for 20 min, and subjected to fluorography.

Digestion with Endoglycosidase F

Samples from the metabolically labeled COS-1 cells transfected with the TGF-β type I receptor cDNA or from PAE/TβR-I cells which were subjected to affinity cross-linking with ^{125}I-TGF-β1 (see below), were immunoprecipitated by the VPN antiserum. The samples were then incubated with 0.5 U of endoglycosidase F (Boehringer Mannheim Biochemica) in a buffer containing 100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% Triton X-100, 0.1% SDS and 1% β-mercaptoethanol at 37°C for 24 h. Samples were eluted by boiling for 5 min in the SDS-sample buffer, and analyzed by SDS-

polyacrylamide gel electrophoresis and fluorograpy for the metabolically labeled cells or autoradiography for the cross-linked cells.

Iodination of TGF-β1, Binding and Affinity Crosslinking

Recombinant human TGF-β1 was obtained from Dr. Arlen Thomason (Amgen Corp.) and Dr. Hideya Ohashi (Kirin Brewery Co.). TGF-β1 was iodinated using the chloramine T method according to Frolik et al. (1984). Cross-linking experiments were performed as previously described (Ichijo et al., 1990). Briefly, cells were washed with binding buffer (phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$ and 1 mg/ml bovine serum albumin (BSA)), and incubated on ice in the same buffer with ^{125}I-TGF-β1 in the presence or absence of excess unlabeled TGF-β1 for 3 h. Cells were washed and cross-linking was done in the binding buffer without BSA together with 0.14 mM of disuccinimidyl suberate (DSS; Pierce Chemical Co.) for 15 min on ice. The cells were harvested by the addition of 1 ml of detachment buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 0.3 mM PMSF). The cells were pelleted by centrifugation, then resuspended in 50 µl of solubilization buffer (125 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Triton X-100, 0.3 mM PMSF, 1% Trasylol) and incubated for 40 min on ice. Another centrifugation was done and supernatants were subjected to analysis by SDS-gel electrophoresis using 4-15% polyacrylamide gels, followed by autoradiography. For immunoprecipitation of the cross-linked complexes, the supernatants were incubated with 7 µl of preimmune serum or VPN antiserum in the presence or absence of 10 µg of peptide for 1.5 h at 4°C. Immune compexes were then added with 50 µl of protein A-Sepharose slurry and incubated for 45 min at 4°C. The protein A-Sepharose beads were washed four times with the washing buffer, one time with distilled water, and the samples were analyzed by SDS-gel electrophoresis using 4-15% polyacrylamide gradient gels and autoradiography.

Northern Blot Hybridization

A Northern blot filter with mRNAs from different human tissues was obtained from Clontech. The filter was hybridized with a ^{32}P-labeled probe at 42°C overnight in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate, pH 6.9, and 0.1 mg/ml salmon sperm DNA. An EcoR1 fragment of 980 bp of the full-length cDNA clone, corresponding to the C-terminal part of the kinase domain and 3' untranslated region (nucleotides 1259-2232 in Fig. 1B) was radiolabeled by the Megaprime DNA labeling system, and used as a probe. The filter was washed two times in 0.5 x SSC, 0.1% SDS at 55°C for 15 min.

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Figure Legends

Figure 1. cDNA Cloning and Sequence of a TGF-β Type I Receptor

(A) Structure of the cDNA clone EMBLA. Boxes represent the coding region; filled box indicates the signal peptide, shaded box the transmembrane domain, and hatched box the intracellular kinase domain. The EcoRI cleavage sites are also indicated. (B) The nucleotide and deduced amino acid sequences of EMBLA. Nucleotides and deduced amino acids are numbered to the right, starting with the proposed initiating Met codon. The N-terminal hydrophobic signal sequence and transmembrane domain are overlined (thin lines), and the potential N-glycosylation site is underlined (thick line). Cysteine residues in the extracellular domain are boxed and the stop codon which ends the open reading frame is marked with an asterisk. The beginning and the end of the kinase domain are indicated by arrows. Half arrows indicate the regions in the kinase domain that were used for construction of the primers for PCR. The in-frame stop codon found in the 5' untranslated region is underlined with a thin line. (C) Comparison of the amino acid sequences of the TGF-β type I receptor (TβR-I) and the human TGF-β type II receptor (TβR-II) (Lin et al., 1992). Alignment was perfomed with the Clustal computer alignment program (Higgins and Sharp, 1989), with some manual adjustment. Identical amino acids are boxed, and cysteine residues in the extracellular domain are shaded. The hydrophobic leader sequence and transmembrane domain of the TGF-β type I receptor are overlined. The borders of the kinase domain are indicated by arrows and the kinase inserts are underlined by bold lines. The kinase subdomains are shown with roman numerals according to Hanks et al. (1988).

Figure 2. Transient Expression of the TGF-β Type I Receptor in COS Cells

Control COS-1 cells and COS-1 cells transfected with the pSV7d expression vector containing the TGF-β type I receptor cDNA were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 6 h. The cell lysates were then subjected to precipitation using preimmune serum (pre) or the VPN antiserum (immune). Blocking of the immune serum was performed with 10 μg of peptide (block). Enzymatic deglycosylation of the TGF-β type I receptor was performed by incubating the immunoprecipitates with 0.5 U of endoglycosidase F (endo F) at 37°C for 24 h. Precipitates were analyzed by SDS-gel electrophoresis and fluorography. Specific bands are indicated by arrows.

Figure 3. Binding of ^{125}I-TGF-β1 to PAE/TβR-I Cells

The parental PAE cells and PAE/TβR-I cells were affinity labeled with ^{125}I-TGF-β1, in the presence or absence of excess unlabeled TGF-β1 (cold TGF-β). After cross-linking with DSS, samples were analyzed by SDS-gel electrophoresis and autoradiography before or after immunoprecipitation by the VPN antiserum (im) or preimmune serum (pre).

Figure 4. Characterization of the TGF-β Type I Receptor in PAE/TβR-I Cells

(A) Enzymatic deglycosylation of the cross-linked complexes by endoglycosidase F. The PAE/TβR-I cells were labeled with ^{125}I-TGF-β1 and cross-linked with DSS. The samples were then treated with 0.5 U of endoglycosidase F (endo F) before or after immunoprecipitation (IP) by the VPN antiserum, and analyzed by SDS-gel electrophoresis and autoradiography. (B) Binding of ^{125}I-TGF-β1 to DTT treated PAE/TβR-I cells. The PAE/TβR-I cells were treated with 1 mM DTT at 37°C for 8 min in the binding buffer without BSA, then incubated with ^{125}I-TGF-β1 followed by cross-linking with DSS. The cross-linked complexes were analyzed by SDS-gel electrophoresis and autoradiography before or after immunoprecipitation (IP) by the VPN antiserum.

Figure 5. Northern Blot Analysis of TGF-β Type I Receptor mRNA

A blot with mRNA prepared from different human tissues (Clontech) was hybridized with a radiolabeled 980 bp internal EcoRI fragment of the TGF-β type I receptor cDNA. Each lane contained 2 μg of polyadenylated RNA from the indicated tissues. An autoradiography of the hybridized blot is shown.

CLAIMS

1. An isolated nucleic acid molecule which codes for, or is complementary to a nucleic acid molecule which codes for, a TGF-ß-type I receptor.

2. A nucleic acid molecule comprising a heterologous sequence which codes for, or is complementary to a nucleic acid molecule which codes for, a TGF-ß-type I receptor.

3. A molecule according to claim 1 or claim 2, wherein the receptor is as shown in Fig. 1(C), or a functionally-equivalent part thereof.

4. A DNA molecule according to any preceding claim.

5. A molecule according to any preceding claim, which additionally comprises, operably associated with the coding sequence, a sequence adapted to allow expression of the receptor.

6. A host comprising a molecule according to claim 5, which is capable of expressing the receptor.

7. An isolated protein having TGF-ß-type I receptor functionality.

8. An isolated protein having an amino-acid sequence corresponding to part or all of the amino-acid sequence of a TGF-ß-type I receptor, wherein said protein has at least one of the following characteristics:

(i) serine/threonine kinase activity;

(ii) TGF-ß binding activity; and

(iii) TGF-ß-type II receptor interaction.

9. A protein according to claim 7 or claim 8, having all or part of the sequence shown in Fig. 1(C).

10. An antibody to a protein as defined in any of claims 7 to 9.

11. A product according to any preceding claim, for therapeutic or diagnostic use.

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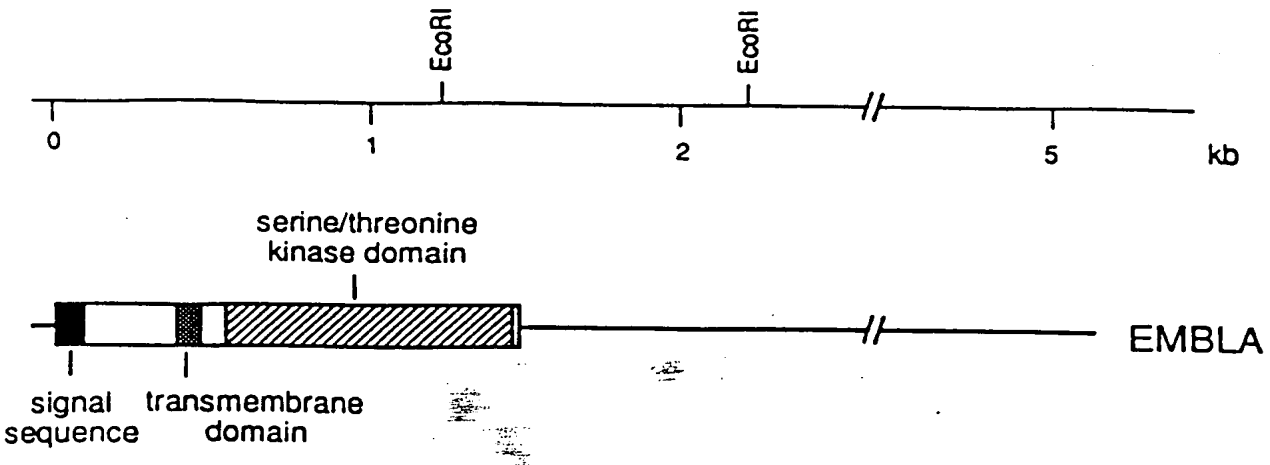
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transmembrane
domain

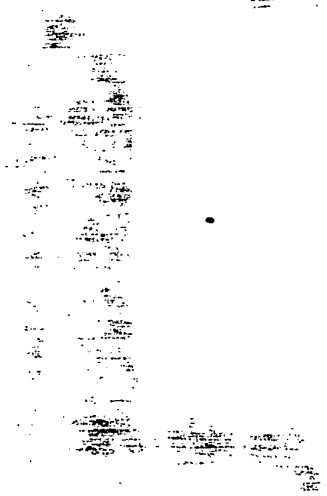

Fig. 1A

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Fig. 1B

C

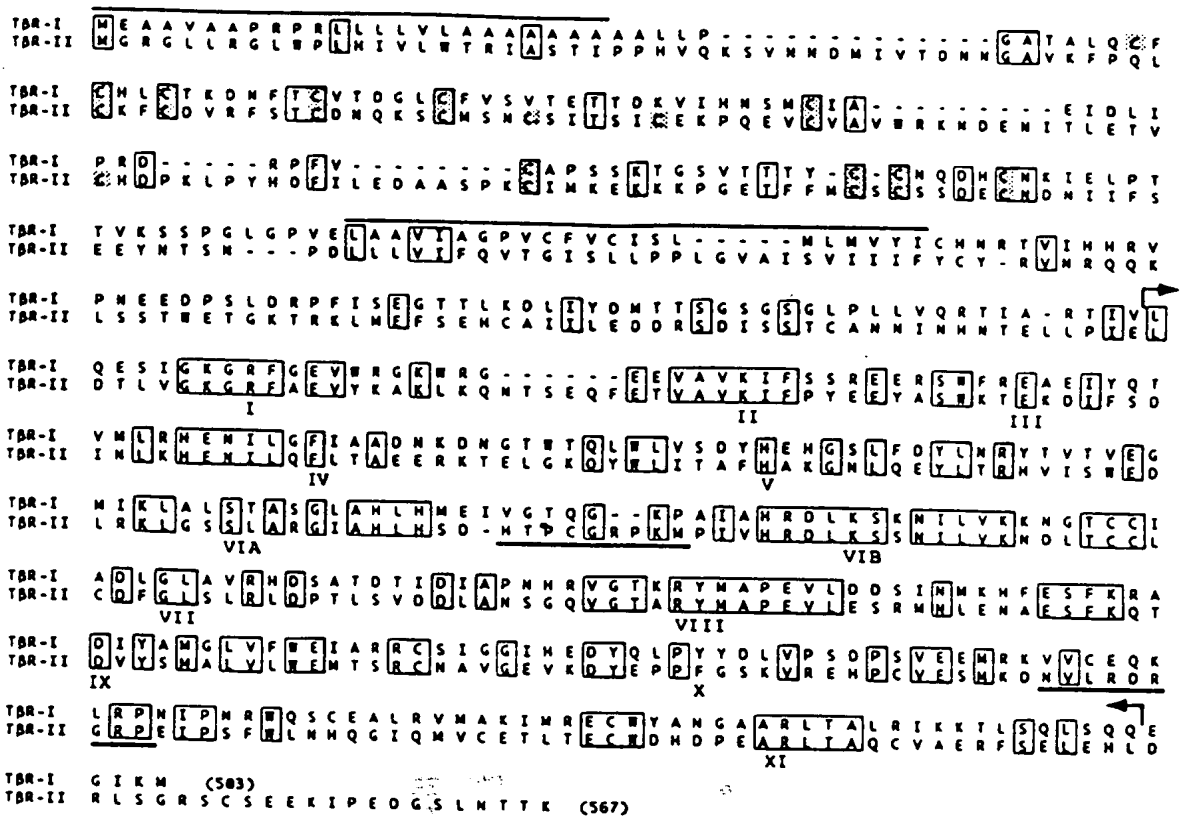
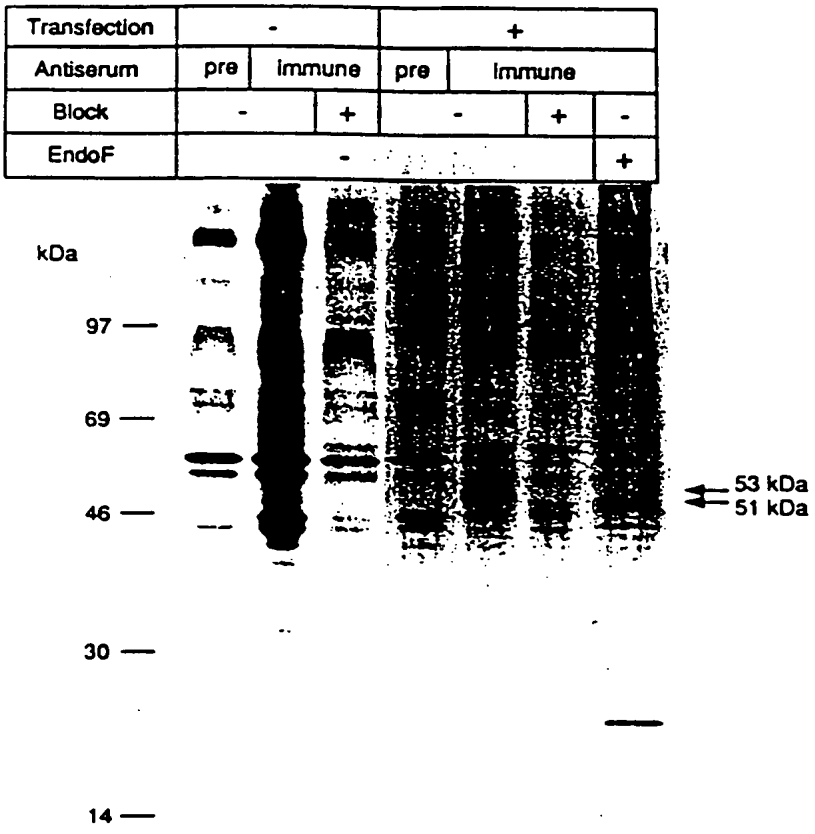


Fig. 1C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Antiserum
pre
Immune
pre
Immune
Block
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+
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+
-
EndoF
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+
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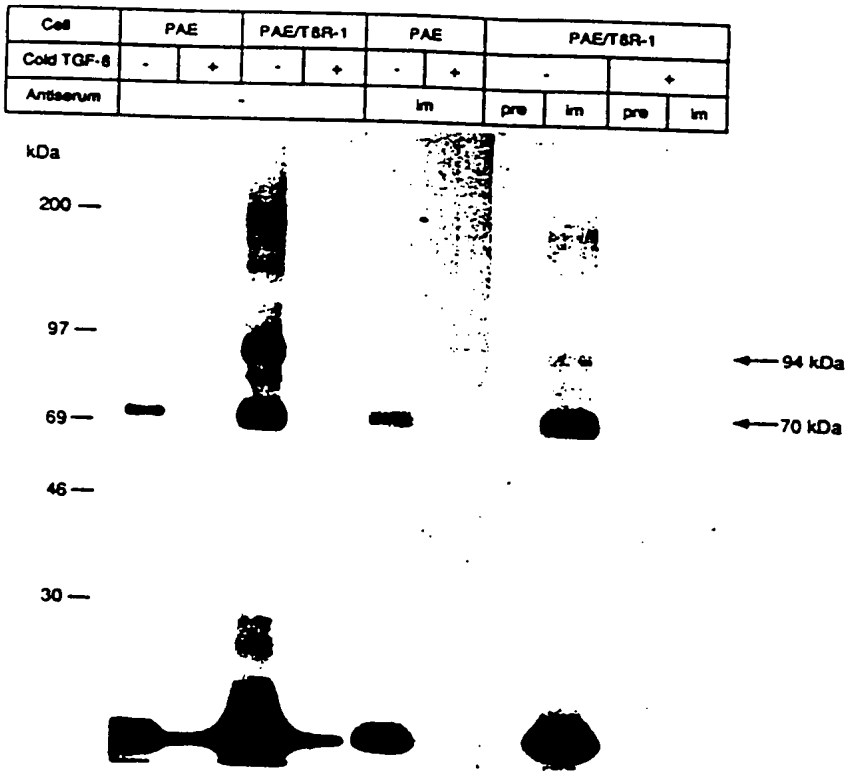
30
14
53 kDa
51 kDa

Fig 2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PAE/T8R-1
PAE
PAE/T8R-1
Cold TGF-ß
-
+
-
+
-
+
-
+
Antiserum
-
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pre
im
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kDa
200
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69
46
30
94 kDa
70 kDa

Fig.3

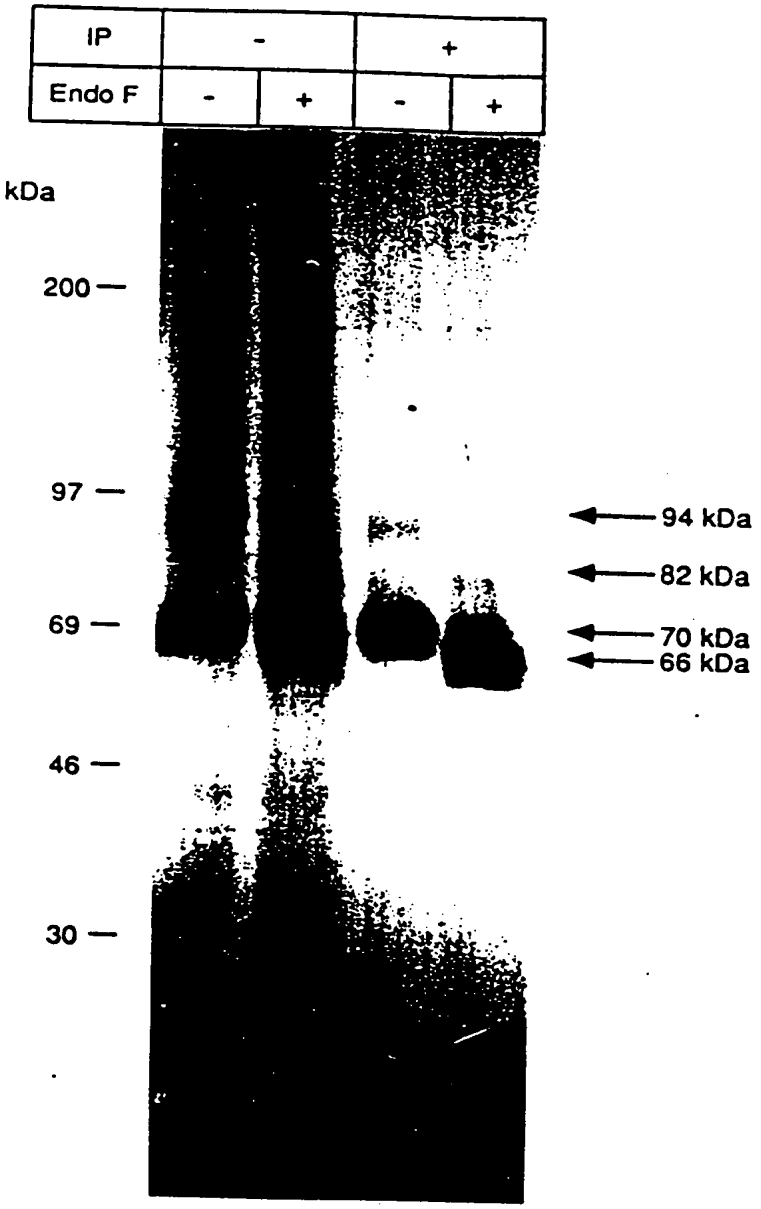

Fig. 4A

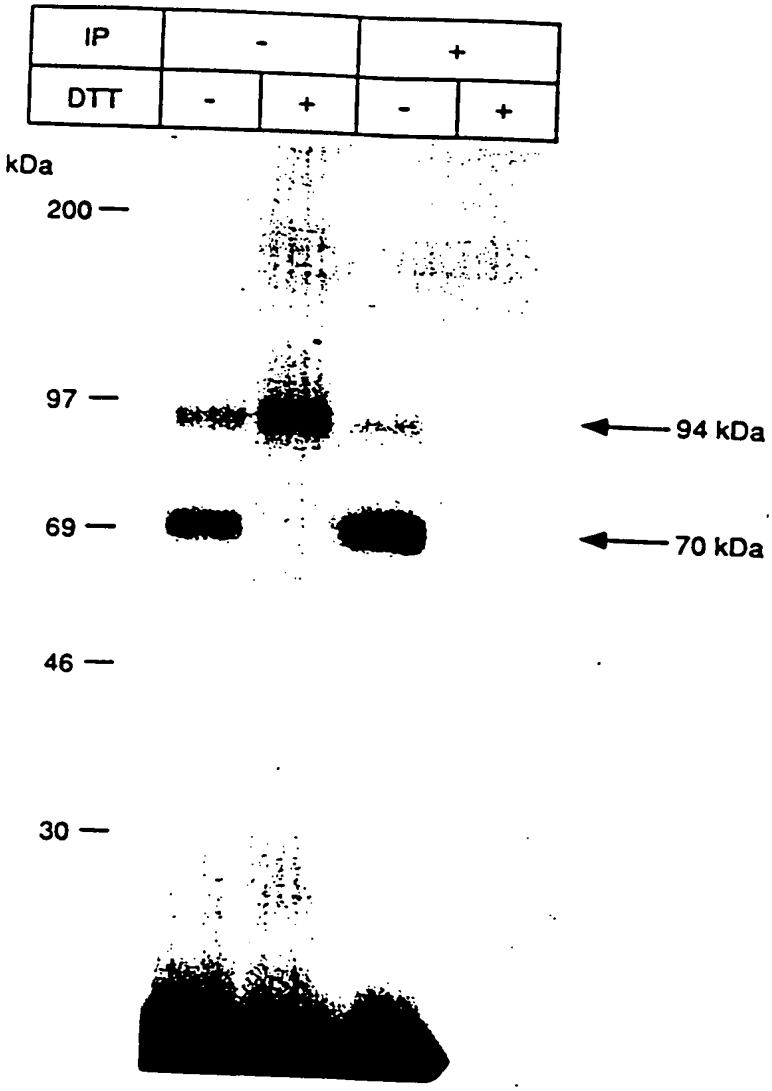

Fig. 4B

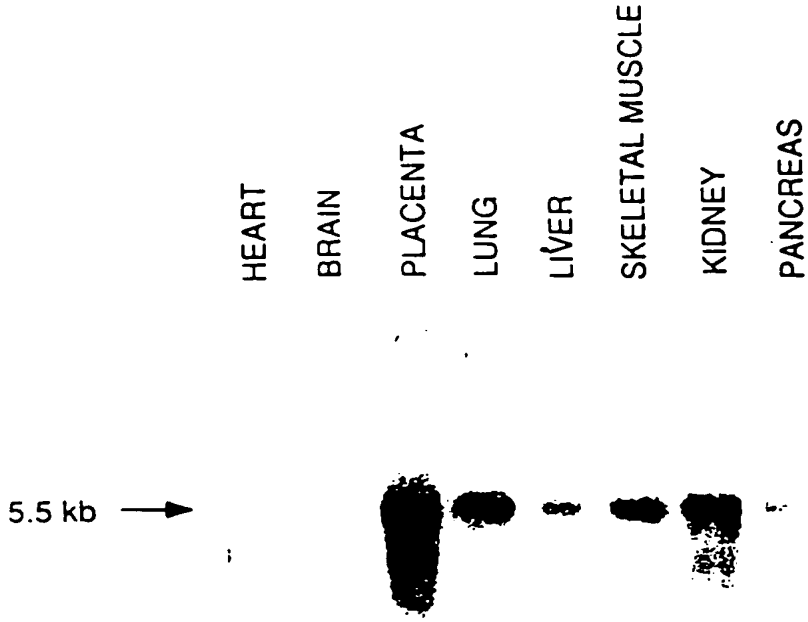
HEART
BRAIN
PLACENTA
LUNG
LIVER
SKELETAL MUSCLE
KIDNEY
PANCREAS
5.5 kb

Fig 5